Engineering of the albumin-FcRn interaction

Thesis for the degree of Philosophiae Doctor

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Abbreviations

β2m	β-2-microglobulin
C-terminal	Carboxyl terminal
C34	Cysteine 34
DI	Domain I
DII	Domain II
DIII	Domain III
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPO	Erythropoietin
Fc	Fragment crystallisable
FcγR	Fcγ Receptor
FcRn	The Neonatal Fc Receptor
FSH	Follicle Stimulating Hormone
GST	Glutathione-S-Transferase
HC	Heavy Chain
HEK	Human Embryonic Kidney cell line
HMEC-1	Human dermal Microvasculature Endothelial Cell line
HSA	Human Serum Albumin
HULEC-5A	Human Lung Endothelial Cell line
IC	Immune Complex
Ig	Immunoglobulin
IgG-IC	IgG-Immune Complex
IMCD	Inner Medullary Collecting Duct
MDCK	Madin Darby Canine Kidney
MHC	Major Histocompatibility Complex
MSA	Mouse Serum Albumin
N-terminal	Amino terminal
SPR	Surface Plasmon Resonance
WT	Wild type

List of publications

Papers included in the thesis

I. Kine Marita Knudsen Sand, **Malin Bern**, Jeannette Nilsen, Bjørn Dalhus, Kristin Støen Gunnarsen, Jason Cameron, Algirdas Grevys, Karen Bunting, Inger Sandlie and Jan Terje Andersen
Interaction with both domain I and III of albumin is required for optimal pH-dependent binding to the neonatal Fc receptor (FcRn)
*Journal of Biological Chemistry, **289**, 34583-34594 (2014)*

II. Jeannette Nilsen, **Malin Bern**, Kine Marita Knudsen Sand, Bjørn Dalhus, Inger Sandlie and Jan Terje Andersen
Human and mouse albumin bind their respective neonatal Fc receptor in different manners
Manuscript

III. **Malin Bern,** Kine Marita Knudsen Sand, Espen S. Bækkevold, Stian Foss, Algirdas Grevys, Jeannette Nilsen, Bjørn Dalhus, Gregory J. Christianson, Derry C. Roopenian, Tilman Schlothauer, Terje E. Michaelsen, Inger Sandlie and Jan Terje Andersen
Engineering albumin for enhanced FcRn-mediated transport across human epithelial cells
Manuscript

Papers related to the thesis

I. Stian Foss, Algirdas Grevys, Kine Marita Knudsen Sand, **Malin Bern**, Pat Blundell, Terje E. Michaelsen, Richard J. Pleass, Inger Sandlie and Jan Terje Andersen.
Enhanced FcRn-dependent transepithelial delivery of IgG by Fc-engineering and polymerization
*Journal of Controlled Release, **223**, 42-52 (2016)*

II. Algirdas Grevys, **Malin Bern**, Stian Foss, Diane Bryant Bratlie, Anders Moen, Kristin Støen Gunnarsen, Audun Aase, Terje E. Michaelsen, Inger Sandlie and Jan Terje Andersen.
Fc engineering of human IgG1 for altered binding to the neonatal Fc receptor affects Fc effector functions
*The Journal of Immunology, **194**, 5497-5508 (2015)*

III. Kine Marita Knudsen Sand, Bjørn Dalhus, Gregory J. Christianson, **Malin Bern**, Stian Foss, Jason Cameron, Darell Sleep, Magnar Bjørås, Derry C. Roopenian, Inger Sandlie and Jan Terje Andersen.
Dissection of the neonatal Fc receptor (FcRn)-albumin interface using mutagenesis and anti-FcRn albumin-blocking antibodies
*The Journal of Biochemical Chemistry, **289**, 17228-17739 (2014)*

IV. Stian Foss, Ruth E. Watkinson, Algirdas Grevys, Martin B. McAdam, **Malin Bern**, Lene S. Høydal, Bjørn Dalhus, Terje E. Michaelsen, Inger Sandlie, Leo C. James and Jan Terje Andersen.
TRIM21 immune signalling is more sensitive to antibody affinity than its neutralization activity
*The Journal of Immunology, **196**, 3452-3459 (2016)*

Part of the introduction is based on the following reviews and book chapter:

I. **Malin Bern**, Kine Marita Knudsen Sand, Jeannette Nilsen, Inger Sandlie and Jan Terje Andersen
The role of albumin receptors in regulation of albumin homeostasis: Implications for drug delivery
Journal of Controlled Release, ***211****, 144-162 (2015)*

II. Kine Marita Knudsen Sand, **Malin Bern**, Jeannette Nilsen, Hanna Theodora Noordzij, Inger Sandlie and Jan Terje Andersen.
Unraveling the interaction between FcRn and albumin: opportunities for design of albumin-based therapeutics
Frontiers in Immunology, ***5****, 1-21 (2015)*

III. Jeannette Nilsen, Kine Marita Knudsen Sand, **Malin Bern**, Peng Lei, Inger Sandlie and Jan Terje Andersen.
The influence of FcRn on albumin-fused and targeted drugs
Springer. Albumin in medicine. Chapter 10 (2016)

Summary

The blood contains immune cells and proteins that among other protect the body and distribute nutrients to meet the body's acute or daily needs. Antibodies with an enormous range of specificities are on the outlook for potential targets and also to communicate further through their effector functions by binding Fc-receptors on cells or soluble molecules such as complement through the fragment crystallisable (Fc)-regions. Albumin is the most abundant protein in blood, with a concentration of 40 mg/ml in both human and mouse. Due to its high abundance, its presence greatly affects the oncotic blood pressure. Albumin is also a carrier of a plethora of small endogenous insoluble substances such as hormones, fatty acids, toxins, and metals as well as a range of medical drugs. Other functions of albumin are the regulation of blood pH, as well as executing enzymatic reactions that may for instance convert prodrugs to active drugs in plasma.

A common feature for immunoglobulin (Ig) G type of antibodies and albumin is that they bind FcRn, and this makes these two unrelated proteins the longest lived serum proteins in humans as serum half-life of both is three weeks. The mechanism behind this longevity is due to a pH-dependent binding to the neonatal Fc-receptor (FcRn) expressed by for instance endothelial cells lining the bloodstream, where binding occurs at pH 6.0 inside acidified endosomes. Binding is followed by recycling to the cell surface, where the neutral pH makes them dissociate from FcRn. However, this mechanism has only been studied for IgG, but as albumin also binds FcRn in the same pH-dependent manner to a distinct site in a non-cooperative manner, it is assumed that the mechanism also applies for albumin.

Antibodies and albumin are also found at the surfaces of the body's largest physical barriers; mucosal surfaces lining the respiratory-, gastrointestinal- and reproductive tracts. Epithelial cells that form such barriers express FcRn that can shuttle IgG across the layer. This cellular process is called transcytosis, and as for recycling, it is based on pH-dependent binding to the receptor in endosomes. As albumin is currently exploited as a drug carrier for half-life extension and for cancer-tissue targeting of therapeutics, it is timely to investigate how FcRn on mucosal surfaces handles albumin.

Furthermore, engineering of albumin-domain III (DIII) and IgG-Fc is a field of interest, in which the aim is to modulate FcRn binding for the tailoring of half-life or transport properties. Novel binders may be used as drug carriers, however, binding and transport properties may vary for each unique fusion or mutant and must be tested *in vitro* before *in vivo* studies. An additional level of complexity arises from the finding that there are large cross-species differences between the FcRn interaction to both IgG and albumin, and such differences must also be considered before *in vivo* studies.

In Paper I and II, the aim was to investigate the contribution of domain I (DI) of albumin in the interaction with FcRn. Mutants with single amino acid substitutions in DI were made and tested for binding to the receptor using *in vitro* interaction assays. These results, in combination with structural inspection of the co-crystal structures of albumin-FcRn, indeed show a contribution of DI, and these interactions strengthen the binding to the receptor.

In Paper II, hybrid molecules of albumin were made, where DI or loops in DI were swapped between the mouse and human forms, followed by testing for binding to FcRn *in vitro*. This was done to investigate if there are differences in the DI-contribution between the species. The results revealed that loss of DI and domain II (DII) in mouse serum albumin (MSA) only slightly hamper binding to mouse FcRn compared to full-length MSA, while introduction of human DI improved binding to both human and mouse FcRn. In addition, human serum albumin (HSA) with mouse DI showed reduced binding to human FcRn compared to complete HSA. These results suggest that the human molecule has evolved to interact with FcRn involving both DIII and DI, while DI plays a minor role for the mouse pair.

In Paper III, we investigated transcytosis of albumin using an *in vitro* transcytosis assay previously used to study FcRn-dependent transcellular transport of IgG. This assay is based on human epithelial cells with endogenous expression of FcRn. Our results demonstrate that albumin is indeed transported, and that efficient transport is dependent on FcRn binding. Furthermore, transcytosis was superior in the apical to basolateral direction. We further made a panel of engineered HSA variants with 5 to 1,500 fold improved FcRn binding at pH 6.0 and variants with weak or strong binding at pH 7.4. Engineered variants with increased binding at acidic pH but not at neutral pH were shown to be transported

considerably more efficient than wild type (WT) HSA. Such mutants may be utilized as carrier of therapeutics for mucosal delivery.

Introduction

The discovery of FcRn

During the 1960s, F.W. Rogers Brambell was the first to postulate that a specific cellular receptor is responsible for active transport of IgG from mother to fetus across the neonatal intestine in rats, or yolk sac in rabbits[1,2]. This theory was based on several observations, demonstrating that during the first 18-21 days of newborn rats, IgG derived from the mother's milk was absorbed by the gut lumen of the suckling rats followed by a rapidly decreased adsorption[3,4]. In addition, using an *in vitro* intestinal assay based on measuring transport of serum proteins across excised intestines from new born rats, this delivery mechanism was shown to be applicable for the IgG isotype and that the Fc-part was essential[5]. In parallel to the discovery of FcRn in neonatal life, it was demonstrated that IgG was eliminated in a concentration-dependent manner from the blood circulation, as injection of high doses of IgG, but not IgA, IgM or albumin increased clearance of IgG in mice[6]. The resemblance of these results and Brambells own made him propose a common receptor for the materno-fetal transport as well for the half-life regulation of IgG[2,7]. Interestingly, Schultze and Heremans proposed in the 1960s that albumin half-life and its concentration dependent catabolic rate were similar to IgG, based on studies on the relative catabolic rates of IgG and albumin in patients suffering from agammaglobulinemia and analbuminemia[8].

Almost two decades later, the actual receptor was identified when it was isolated from the rodent gut as a heterodimeric protein consisting of two subunits of 40-46 kDa and 12 kDa[9]. In a follow-up study the rodent FcRn-genes were cloned, which revealed that the 40-46 kDa heavy chain (HC) was related to the major histocompatibility complex (MHC) class I HCs and that the smaller 12 kDa subunit was β-2-microglobulin (β2m)[10]. Hence, "neonatal" is inspired by its discovery.

A few years later, the human orthologue of FcRn was cloned from the placenta derived syncytiotrophoblasts[11], and it was found that it was a parallel mechanism for the transport of humoral immunity to offspring in humans, however, in this case transport happens pre-birth[12]. Specifically, the direct transcytosis of mothers IgG to fetus occurs during the third trimester of pregnancy[12]. Importantly, the binding of IgG to FcRn was found to be strictly pH-dependent, meaning that

binding only occurs at acidic pH and no binding but release at neutral pH[11,13]. Of notice, later studies in humans and rodents revealed that FcRn expression is not restricted to neonatal life, but that it is broadly expressed in a number of cell types throughout the body, and its functions involves recycling and transcytosis[14–34]. A hallmark for all known functions of FcRn is that it binds IgG pH-dependently. This strict pH-dependence translates into a cellular transport model where IgG is taken up from the exterior followed by binding to FcRn as a function of the acidified environment found within endosomes and exocytosis to the cell surface where IgG is released when exposed to the neutral pH (Figure 1)[14,35–38].

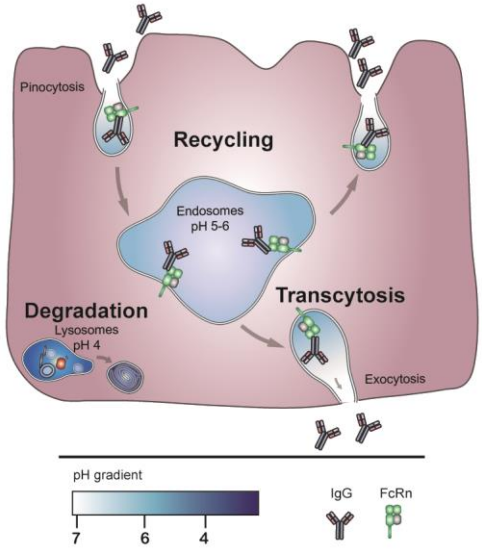

Figure 1. Models of FcRn mediated cellular transport pathways of IgG. FcRn mediated transcytosis and recycling of IgG in an epithelial or endothelial cell lining the luminal side or blood, respectively. IgGs are taken up by fluid-phase pinocytosis from the exterior. When in the acidicified endosomes, FcRn will bind IgG followed by transcytosis to the basolateral side or recycling back to the blood in an epithelial or endothelial cell, respectively. Here, the neutral pH will trigger release of IgG.

In addition, a new ligand for FcRn was identified over a decade ago, when Anderson and colleagues co-eluted a 67 kDa protein together with human FcRn from an IgG coupled column, which was found to be bovine albumin[39]. Interestingly, as for IgG, the interaction was shown to be strictly pH-dependent and that both ligands can engage the receptor simultaneously, bind in a non-cooperatively manner, and as such that the same receptor is responsible for their unique long serum half-lives[40,41]. The consequences of albumin binding to FcRn are gradually emerging, and it is now been shown that FcRn expressed in proximal tubular cells of the kidneys are involved in albumin reabsorption from the urinary filtrate, which was the first evidence of a role of FcRn in transcellular transport of albumin[42–44]. Additionally, other albumin receptors such as cubilin and megalin have also been shown to be involved in albumin retrieval and may act in concert with FcRn at such sites[45–50]. Exactly how FcRn handles albumin at the different sites in the body remains unknown.

FcRn rescues albumin and IgG from intracellular degradation

IgG and albumin are structurally and functionally unrelated but share two unique features (1) they are the most abundant serum proteins in blood with a concentration of 12 mg/ml and 40 mg/ml, respectively, and (2) they have a half-life of three weeks in humans, in contrast to other serum proteins with half-lives from hours up to a few days only. While IgG is produced and secreted by B lymphocytes, albumin is exclusively produced by hepatocytes of the liver. The rate of synthesis and secretion of albumin is primary regulated by the oncotic pressure detected by osmoreceptors in the hepatic interstitium[51], while production of IgGs occurs in response to foreign substances that trigger their production from B lymphocytes. In general, half-life of serum proteins are determined by the rate of synthesis, susceptibility to enzymatic degradation and its size, as the renal clearance threshold is 60-70 kDa.

The first proof of FcRn as a homeostatic regulator of IgG was shown in mice deficient for the β2m subunit of FcRn, where a functional FcRn cannot be produced, and greatly reduced levels of endogenous and injected IgG were observed[19]. The first demonstration for the involvement of FcRn in albumin homeostasis was in mice knock out for the FcRn HC where 2-3 fold lower albumin levels were detected compared with normal mice[39]. The same mice showed 4-5 fold lower IgG levels, where the more drastic drop in levels of IgG compared to albumin was found to be due to increased albumin synthesis[39,52]. Further support for the involvement of FcRn in half-life regulation is given by humans carrying a single-point mutation in the gene encoding β2m, which have 80-90% lower expression levels of FcRn and consequently very low serum levels of IgG and albumin, a rare syndrome named familial hypercatabolic hypoproteinemia[53,54]. Recently, a new case of this syndrome was identified, where two β2m deficient siblings were found to have a novel homozygous splice site in the B2M gene that caused a frame shift and a premature stop codon, thus, no FcRn expression and low levels of IgG and albumin were also shown in these patients[55].

In light of the fact that FcRn is broadly expressed, it is of interest to dissect which type of cells or organ that contributes to the FcRn-mediated salvage of IgG and albumin. Hematopoietic and vascular endothelial cells have been proven to be important, as mice with conditional deleted FcRn in these cells show 4 fold and 2 fold reduced IgG and albumin, respectively, than normal mice[20,23,56].

At a cellular level, the mechanism for salvage by FcRn has been extensively investigated for IgG using advanced imaging studies and the human microvasculature endothelial cell line, HMEC-1 that overexpress FcRn, as well as the human lung microvasculature endothelial cell line, HULEC-5A with endogenous FcRn expression[14,36–38,57,58]. These imaging studies demonstrate that FcRn is predominantly located within acidified endosomes and that IgG taken up via fluid-phase endocytosis can engage FcRn in the acidified endosomes followed by exocytosis to the cell surface in complete fusion or a prolonged-release manner, where in both cases the neutral pH of the exterior trigger their dissociation[14,36–38,57,58]. This prolonged release mechanism of exocytosis is a novel variant of the so-called kiss-and-run, where the release of IgG occurs in multiple steps in a periodic fashion and the exocytotic vesicle remains structured[36]. In addition, FcRn may diffuse to the plasma membrane and it is suggested that after dissociation, retrieval of IgG can occur[36]. Rab GTPases are intracellular trafficking regulators that regulate many intracellular processes, including endosome trafficking, and in the process of exocytosis. For instance, Rab5 is an early endosomal marker, while Rab4 and Rab11 are involved in recycling to the plasma membrane from sorting endosomes[59]. FcRn-containing sorting endosomes have been shown to be first associated with Rab4 and/or Rab11, however, only Rab11 is present on the exocytotic vesicles during plasma membrane fusion[58]. Of notice, proteins that do not bind FcRn are sorted to the lysosomal degradation pathway[14,37].

As for now, there are no direct reports on the intracellular trafficking events of albumin-FcRn-containing endosomes, however, *in vitro* studies show that albumin and IgG can bind simultaneously to FcRn in a non-cooperatively manner, and both pH-dependently[39,41]. These studies suggest that albumin may follow the same route of intracellular transport. Hence, the intracellular events that ensure albumin longevity remain to be studied. In addition, as the same cell-types are involved in the recycling of both albumin and IgG, proof for the formation of the ternary complex of IgG, FcRn and albumin in a cellular setting, and if this occurs *in vivo* remains to be resolved. A schematic model of the cellular recycling salvage mechanism of albumin and IgG is shown in figure 2A.

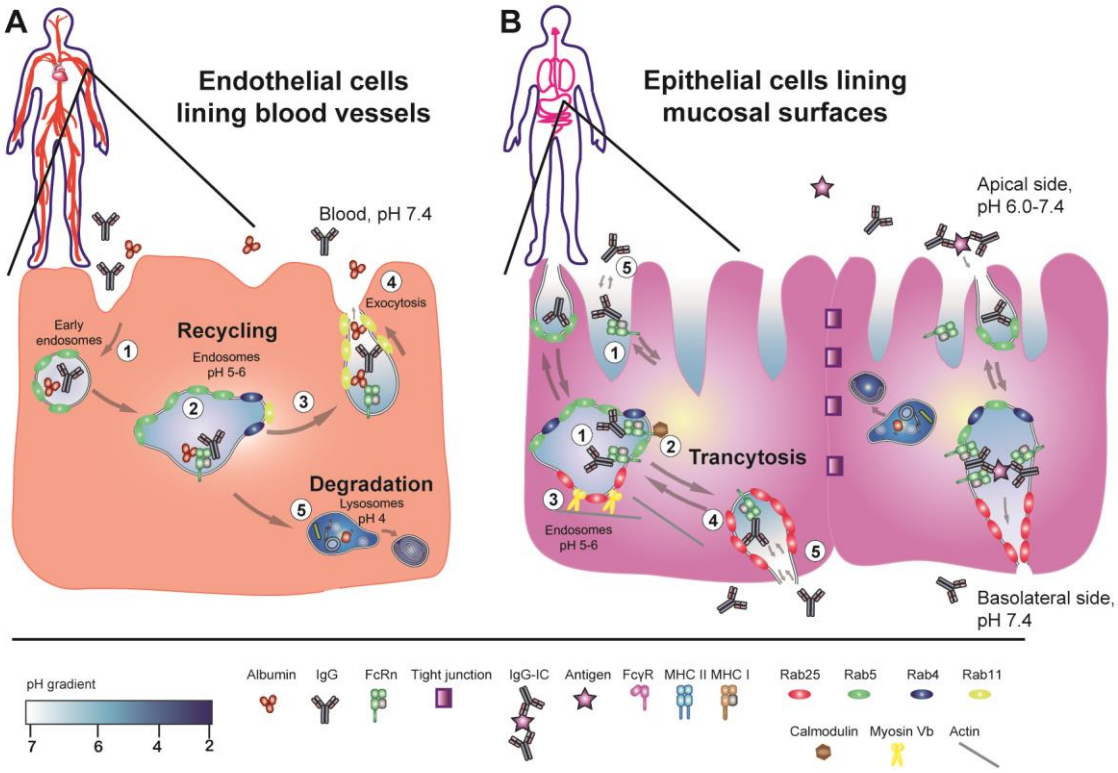

Figure 2. FcRn mediated recycling and transcytosis. A. A schematic drawing of the FcRn-mediated salvage mechanism of albumin and IgG in endothelial cells lining the blood. Albumin and IgG are taken up by fluid-phase pinocytosis into Rab5-positive early endosomes (1). When encountering FcRn in Rab4 and Rab11 positive recycling compartments, the low pH allows for binding of both ligands to the receptor (2). The ternary complex will be transported to the cell surface where exocytosis from Rab11 positive vesicles or tubules can occur (3), and the neutral pH of the blood causes their dissociation from the receptor (4). Proteins that do not bind FcRn are sorted to the lysosomal degradation pathway (5). **B.** An illustration of FcRn-mediated transcytosis of IgG and IgG-ICs across polarized epithelial cells. IgG can bind FcRn in acidic endosomes or possibly at acidic epithelial surfaces (apical) (1). Calmodulin-binding to the cytoplasmic tail of FcRn (2), as well as the engagement of Myosin Vb (3) and Rab25 (4) are involved in the transcytosis. Fusing with the basolateral or apical membrane causes exocytosis of IgG as the neutral pH triggers dissociation (5). Monomeric IgGs can be transcytosed from the basolateral side to the apical side for subsequent binding to their cognate antigen, followed by transcytosis of the generated IgG-IC to the basolateral side.

FcRn mediates transcytosis of IgG at mucosal surfaces

FcRn is expressed in epithelial cells lining the mucosal surfaces of the body including the gastrointestinal tract, respiratory tract and female vaginal tract where FcRn has been shown to mediate bidirectional transport of IgG[27,29–31]. Using an *in vitro* cellular assay by growing Madin Darby canine kidney (MDCK) cells overexpressing human FcRn on transwell filters, it was shown that FcRn can

transcytose IgG from either the basolateral or apical side to the opposite side[60,61]. In addition, results from other *in vitro* cellular transcytosis systems using the cell lines; rat Inner Medullary Collecting Duct (IMCD) cells, human intestinal CaCo-2 or T84 cells and BeWo or JAR cells derived from placenta[30,62–66], *in vivo* models using mice, non-human primates and humans[27,67–71], as well as *ex vivo* placenta transfer models[12,72,73] support that FcRn mediates transcytosis of IgG across epithelial cells. The cytosolic calmodulin, GTPase Rab25 and the actin motor myosin Vb have been shown to regulate the transcytosis determination across polarized MDCK cells, after uptake from either the basolateral or apical side into recycling endosomes[61,74]. In addition, the cytoplasmic tail of FcRn HC contains conserved di-leucine (L322/L323) and tryptophan (W311) sorting motifs that interact with the adaptor protein-2 σ-γ subunits and μ subunit, respectively, and are important for the rapid endocytosis of the receptor and basolateral targeting in polarized rat cells[75,76]. Two phosphorylation sites are also present in the cytoplasmic tail of FcRn, where one (S313) has been shown to be involved in apical to basolateral transcytosis in rat IMCD cells[77].

Only one report has investigated the direct involvement of FcRn in transcytosis of albumin, by using MDCK cells overexpressing rat FcRn[78]. The rationale for this study was to address if the stoichiometry affects cellular trafficking of the ligands as FcRn binds the homodimeric IgG in 2:1 manner, while albumin binds FcRn by 1:1[39,79]. However, while IgG was shown to be transported, no FcRn-dependent transport of rat albumin was measured[78].

It is interesting that both albumin and IgG interact with FcRn and that both ligands are found at mucosal surfaces[80]. Specifically, albumin has been found in several mucosal secretions like saliva (1-3 μg/ml), feces (100 μg/ml) and from the small intestine (500 μg/ml)[80]. This encourages the investigation of how albumin is distributed at such sites. The involvement of FcRn and how transport of one ligand affects transport of the other in epithelial cells is investigated in Paper III.

Structural inspection of the FcRn-ligand interactions

The essence of the interactions of both IgG and albumin with FcRn is that they are pH-dependent, with binding only at acidic pH (pH 5.0-6.0), and no binding or dissociation at neutral pH (pH 7.4). Due to the unique biochemical properties of histidines, the strict pH-dependence relies on the protonation of imidazole groups when reaching neutral pH. The FcRn-IgG interaction has been extensively studied,

however, the complete interactive interface for the FcRn-albumin interaction has not been described.

The HC of FcRn is membrane bound, *N*-glycosylated and folded into three extracellular domains (α1, α2 and α3), followed by a transmembrane segment and a cytoplasmic tail. The amino terminal (N-terminal) α1-α2-platform is folded as eight antiparallel β-strands topped with two α-helices. β2m (12 kDa) is non-covalently associated with the extracellular part of the HC by making contact with the α3-domain that is located below the α1-α2-platform. The main structural difference between FcRn and the MHC class I molecules is that the α1-α2 platform makes up a peptide-binding groove in MHC class I, while this is closed in FcRn, which is a result of a single valine to proline substitution in the α2-helix that causes bending and a repositioning of the α1-helix in FcRn[10,79,81–83].

Albumin (66.5 kDa) is a non-glycosylated heart shaped molecule made up of a single polypeptide chain of 585 amino acids with 17 disulphide bridges and one free cysteine (C34). It is folded into thee homologous domains (DI, DII and DIII) and six sub-domains (DIA, DIB, DIIA, DIIB, DIIIA and DIIIB). The secondary structure is mostly built up by α-helices (67%) and flexible loops connects the domains[84–86].

The amino acids on IgG involved in binding to FcRn have been mapped by inspection of the co-crystal complex of rat FcRn and rat IgG2a Fc, as well as binding studies using IgG mutants for binding to FcRn[82,87–90]. These studies demonstrated key roles of the conserved histidines, H310, H435 as well as I253 located in the so-called elbow region in the Fc-domain of IgG[82,87–90]. The pH-dependent binding is suggested to be due to protonation of H310 and H435 at low pH that can mediate favourable interactions with FcRn, which stabilize the formation of hydrophobic contacts by I523. Similarly, it has been found that protonation of a histidine is involved in the albumin-FcRn interaction. Specifically, it was found that H166 in the α2-domain of human FcRn was essential for binding by *in vitro* interaction assays[40,41], and inspection of two crystal structures of FcRn solved at either acidic pH (pH 4.2) or basic pH (pH 8.2) revealed that this conserved residue is positioned closely to an exposed loop in α1 that is only stabilized when H166 is protonated by the generation of stabilizing hydrogen bonds[79,91,92]. The loop contains four conserved tryptophan residues (W51, W53, W59 and W61), and the impact of these residues was shown experimentally when each of them were mutated to alanine, which gave reduced or no binding[93,94]. As

such, the interaction is not only pH-dependent but also hydrophobic in nature that supports an earlier report using isothermal titration calorimetry[41]. Thus, the two ligands have evolved to bind to separate binding sites on each side of the α1-α2-platform. The binding sites for the ligands on FcRn are highlighted in figure 3A.

The first in-depth report of the FcRn-albumin interaction, presented a docking model based on available crystal structures of singe molecules and *in vitro* interaction-studies using mutant and truncated albumin variants[92]. Specifically, mutation of the three conserved histidines; H464, H510 and H535 to glutamine, as well as lysine on position 500 to alanine in albumin, gave considerable decreased binding[92]. Interestingly, truncated variants of albumin have been tested for FcRn binding, and no binding was detected for DI-DII albumin[92,95], however, binding was seen for DIII albumin, but with a K_D value 10 fold lower than the complete molecule[92]. This supports the hypothesis that DIII is the principle binding-domain. The same study reported that DI-DIII bound somewhat better than DIII, although this molecule may not reflect the natural positions of DI and DIII compared to the full-length. However, the best-fitted docking model suggested that DI could be involved in the interaction via two exposed loops. This encourages further investigation on how DI of albumin is involved in FcRn binding (Paper I and II). Figure of the solved crystal structure of albumin, as well as amino acids identified to be involved in binding to FcRn, are highlighted in figure 3B.

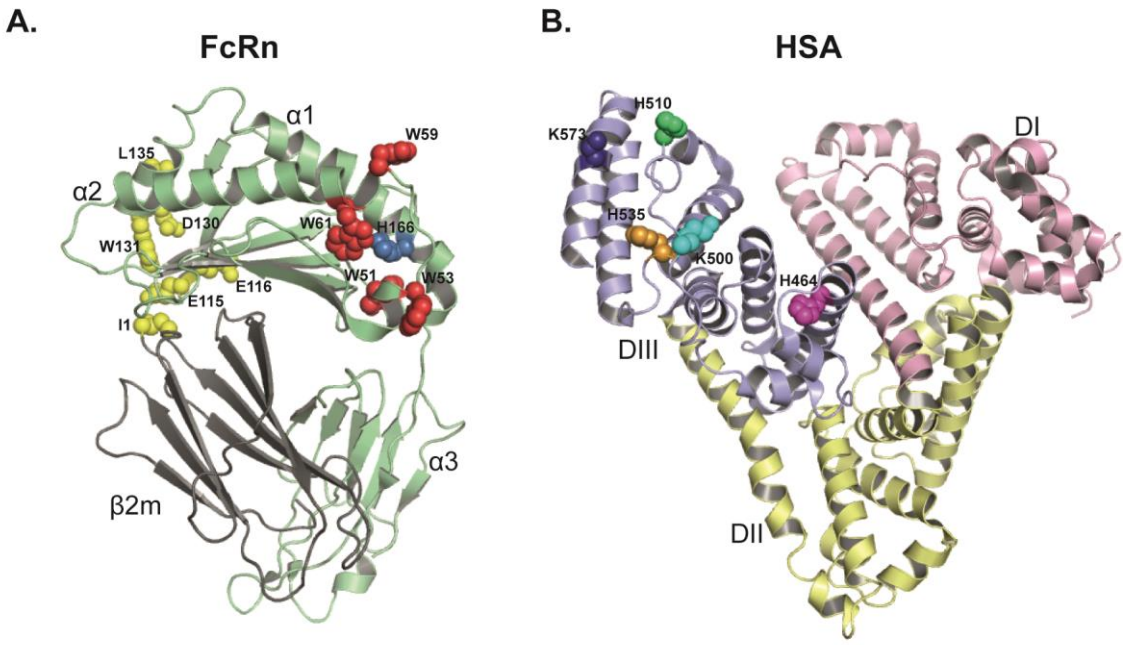

Figure 3. Crystal structures of human FcRn and HSA. A. The solved crystal structure of recombinant soluble human FcRn with its HC (α1, α2 and α3) in green that is non-covalently associated with β2m in grey. Amino acid residues identified to be important for binding to albumin are shown in spheres; H166 in blue and W51, W53, W59 and W61 in red. Amino acid residues found to be important for IgG binding are shown in yellow spheres; E115, E116, D130, W131 and L135, as well as I1 in β2m. **B.** The solved crystal structure of HSA with its DI, DII and DIII in pink, yellow and blue, respectively. Amino acid residues shown to be important for binding to FcRn are shown in coloured spheres; H464 (magneta), K500 (cyan), H510 (green) and H535 (orange). In addition, the amino acid residue K573 is shown in a dark blue sphere. The figures were generated using PyMol (DeLano Scientific) with the crystallographic data of the solved structures of human FcRn[91] (A) and HSA[96] (B).

Engineering of the FcRn-ligand interactions

There are several examples of Fc-engineering of human IgG for altered binding to FcRn that translate into improved *in vivo* half-life and therapeutic efficacy[73,97–102]. One such variant contains three mutations in the CH2 domain of the Fc (M252Y/S254T/T256E, MST), which gave 10 fold improved binding to FcRn at pH 6.0 and 2-4 fold improvement in half-life compared to WT IgG in humans[97,103]. However, when strong binding at both neutral and acidic pH is achieved, release at the plasma membrane is impaired and the antibody will be captured on the receptor inside the cells. One such variant, the Fc-engineered mutant (M252Y/S254T/T256E/H433K/N434F, MST-HN), has been shown to eliminate autoreactive circulating IgGs by blocking binding of such to FcRn in mouse disease models[104–106]. Due to this feature, it is called "antibody for degradation".

As new and detailed knowledge emerge on the albumin-FcRn interaction, there is increased interests in making albumin mutants with altered binding properties that may pave the way for the development of albumin-based therapeutics with improved pharmacokinetics[92,95,107,108]. And as for IgG, the main goal in such design will be to generate mutants with strong binding at acidic pH and no or minor binding at neutral pH for the efficient release at the cell surface. Design of a panel of such variants with different FcRn binding properties and how such mutants behave in a cellular setting are investigated in Paper III.

One recent example of an engineered albumin variant was designed based on knowledge obtained from an investigation of the cross species differences in the binding between human and mice, where it was first shown that MSA binds more strongly to human FcRn than HSA[109]. In a follow-up experiment, a hybrid molecule of HSA exchanged with mouse DIII was designed, and improved binding to both human and mouse FcRn was shown[107]. In addition, the last carboxyl terminal (C-terminal) α-helix of HSA was swapped with MSA, which also increased binding to mouse FcRn compared to HSA[107]. Further investigation of the last C-terminal α-helix focused on a specific proline on position 573 that is conserved in all species, except humans and orangutan, which have a lysine[108] (highlighted in figure 3B). Substitution of this position to all other amino acid in HSA gave improved binding to human FcRn, and the K573P mutant with 12 fold improved binding extended half-life from 2.8 to 4.0 days and 5.4 to 8.8 days in human FcRn transgenic mice and cynomolgus monkeys, respectively[108].

To date, no more than 14 fold improvement of binding has been achieved without breaking pH-dependence in the IgG-Fc engineering field. It would be interesting to investigate how strong it is possible to engineer the albumin-FcRn interaction at acidic pH before the pH-dependence is disrupted, and how such mutants behave in an *in vitro* cellular setting and *in vivo*. The combination of single mutants, each giving a contribution to improved FcRn binding, may affect cellular transport. This is studied in Paper III.

The importance of cross-species differences in FcRn-ligand binding

Pre-clinical studies on the pharmacokinetics of albumin- and IgG-based drugs are most commonly done in rodent models. However, large cross species differences

have been discovered in recent years for binding of both albumin and IgG to FcRn. Specifically, mouse IgGs binds weakly to human FcRn[109,110], thus explaining the short persistence in serum during the first evaluations of mouse monoclonal antibodies in humans[111,112]. On the other hand, mouse FcRn binds more strongly to human IgG[109,110].

Cross-species differences are also seen for the albumin FcRn interaction, as both mouse and human FcRn binds more strongly to MSA than the human counterpart[107,109]. This has tremendous implications when evaluating HSA-based drugs in mice, as the molecules tested may be outcompeted by endogenous MSA. Such studies have been done, and the half-life of HSA in normal mice was found to be similar to a mutant HSA variant (K500A) with 30 fold reduced binding to human FcRn[108]. Interestingly, the K573P mutant show increased half-life compared to HSA from 21 to 30 hours in normal mice, thus illustrating that the mutation improved its competitive abilities due to increased binding to mouse FcRn[108]. However, the half-life of MSA has been shown to be 35-39 hours, and engineered variants with further improved binding would be needed to fully outcompete MSA in normal mice[39].

Further investigation of the structural areas and residues in albumin that differ among species may pave the way for design of human albumin variants with further improvement in pH-dependent binding to FcRn. As such, investigation of mouse-human hybrid albumin variants may reveal how amino acid differences between HSA and MSA affect FcRn binding. Interestingly, a hybrid albumin with human DI-II and mouse DIII bound more strongly than full-length MSA to human FcRn, pointing to a favourable contribution of human DI-DII in the interaction[107]. This encourages further investigation of the influence of DI in binding to mouse and human FcRn, which are addressed in Paper II.

In vitro interaction and cellular assays may be cost-effective methods for screening of engineered albumin variants and their behaviour in regard to FcRn prior to *in vivo* evaluation. As such, only engineered variants with the optimal binding strength at different pH values and desired transport capacity can then be chosen from a larger panel. Of interest, as FcRn is expressed at several mucosal surfaces, studies of FcRn as a delivery vehicle may give the insight necessary to design the next-generation delivery strategies of albumin fused drugs.

Aims of the thesis

1. Albumin is the most abundant protein in blood and due to the binding to FcRn, a long half-life is ensured. To understand the interaction, it is important to investigate the structural elements involved. Inspection of the available docking model of the albumin-FcRn interaction suggests that DI may be involved in binding. However, experimental data to demonstrate how DI contributes is lacking. To investigate the involvement of DI in binding to FcRn, we set out to mutate selected residues in two exposed loops of DI and test the resulting mutants for binding to FcRn *in vitro,* using surface plasmon resonance (SPR) and enzyme-linked immunosorbent assay (ELISA) (Paper I and II).
2. It is well known that there are cross-species differences in the albumin-FcRn interaction between human and mouse. Both mouse and human FcRn bind more strongly to MSA than HSA, and mouse FcRn binds weakly to HSA. The amino acid difference in position 573 of albumin is partly responsible for these differences. Furthermore, it has been reported that a hybrid albumin variant with human DI-II and mouse DIII binds better than MSA to human FcRn, suggesting a favourable contribution of human DI in the interaction. We aimed to investigate the involvement of DI in the FcRn interaction including human and mouse molecules. This was done by making albumin hybrids and mouse and human recombinant DIII, followed by testing for binding to FcRn *in vitro* by SPR and ELISA (Paper II).
3. FcRn is expressed by epithelial cells lining mucosal surfaces, where it mediates bidirectional transcytosis of IgG. Several reports support this mechanism after both *in vitro* cellular assays and *in vivo* studies. In contrast, in a study published over a decade ago, using transfected MDCK cells overexpressing rat FcRn, FcRn-dependent transport of rat albumin was not found and no further investigation has been reported since. However, as albumin and IgG bind FcRn in the same pH-dependent way and in a non-cooperative manner *in vitro*, we aimed to investigate the cellular transport of albumin by using non-manipulated human epithelial cells with endogenous expression of FcRn. In addition, we aimed to design novel engineered HSA variants with increased binding strength beyond that of the K573P mutant. The variants were tested for their pH-dependent binding properties to FcRn *in vitro* by using ELISA and SPR, as well as an FcRn coupled column. The rates of transcytosis for the different variants were compared to investigate if improved pH-dependent binding enhances transcellular transport (Paper III).

Summary of the individual papers

Paper I

Kine Marita Knudsen Sand, **Malin Bern**, Jeannette Nilsen, Bjørn Dalhus, Kristin Støen Gunnarsen, Jason Cameron, Algirdas Grevys, Karen Bunting, Inger Sandlie and Jan Terje Andersen

Interaction with both domain I and III of albumin is required for optimal pH-dependent binding to the neonatal Fc receptor (FcRn)

*Journal of Biological Chemistry, **289**, 34583-34594 (2014)*

In Paper I, we investigated the contribution of HSA DI in human FcRn binding. Constructs of albumin DIII, DI-DII and full-length were tested for their ability to compete with complete albumin for binding to FcRn. Albumin with all three domains competed best, followed by DIII and DI-DII. Furthermore, selected amino acids in two exposed loops of HSA DI were mutated, followed by testing of the mutants for binding to FcRn. Specifically, substituting D108 to alanine showed greatest negative effect on FcRn binding compared to WT with 4.3 fold reduction. On the opposite end, alanine substitution of N109, N111, L112 or P113 improved binding up to 2.8 fold. In addition, two rare natural occurring HSA mutants with mutations in these loops were tested, E82K and R114G, which both showed reduced receptor binding by approximately 2 fold. Moreover, CD and stability analysis showed that the weakest binder (D108A) had 7-8% less α-helices and was less stable than WT. Importantly, inspections of two solved co-crystal structures published during the course of the work confirmed that the two exposed loops of DI are in close proximity and can engage FcRn.

Paper II

Jeannette Nilsen, **Malin Bern**, Kine Marita Knudsen Sand, Bjørn Dalhus, Inger Sandlie and Jan Terje Andersen

Human and mouse albumin bind their respective neonatal Fc receptor in different manners

Manuscript

In Paper II, the contribution of albumin DI in binding to human and mouse FcRn was investigated. HSA DI single mutants, and mouse human hybrid albumin constructs were made; DI-loop-swaps and DI-swaps, as well as DIII constructs. Such molecules were then tested *in vitro* for binding to both human and mouse FcRn. Altered binding properties were seen when mutating K466 in DIII or H105 in DI to alanine, and together with inspections of the co-crystal structures of the albumin-FcRn complex, this suggests a role of these residues in making intramolecular contacts via D108. Of the single mutants made based on targeting of amino acids of the two exposed loops of HSA DI, alanine substitution of E86 and D89 had the greatest negative impact on binding to human FcRn with more than 3 fold reduction. Furthermore, mouse DIII bound 2 fold more weakly than full-length MSA to mouse FcRn, while similar binding strength was shown to human FcRn. Human DI swapped into MSA improved binding to both human and mouse FcRn. In contrast, mouse DI swapped onto HSA caused reduced binding to human FcRn. Swapping of both exposed loops in DI between human and mouse albumin showed the same trend as for the DI swapping for binding to their corresponding receptors, although human FcRn binding of MSA with humanized loops was less influenced. Interestingly, swapping of two residues that differ between human and mouse in the albumin DI-DII interface slightly increased binding to human FcRn in both cases. Taken together, the results suggest a minor role of the mouse DI in the FcRn interaction compared to the human pair. Such knowledge may pave the way for design of DI-engineered albumin variants with novel binding and transport properties.

Paper III

Malin Bern, Kine Marita Knudsen Sand, Espen S. Bækkevold, Stian Foss, Algirdas Grevys, Jeannette Nilsen, Bjørn Dalhus, Gregory J. Christianson, Derry C. Roopenian, Tilman Schlothauer, Terje E. Michaelsen, Inger Sandlie and Jan Terje Andersen

Engineering albumin for enhanced FcRn-mediated transport across human epithelial cells

Manuscript

In Paper III, an *in vitro* transcytosis assay was used for the investigation of albumin transcytosis using the human T84 cell line that express FcRn endogenously. We found that albumin is indeed transcytosed, and that efficient transport depends on the pH-gradient of the endosomal compartments. Furthermore, low rate of transcytosis was measured when treating cells with an antibody that blocks the albumin-binding site on FcRn (ADM31), as well as for a mutant with no measurable binding to FcRn (K500A/H510Q). Further, a panel of engineered albumin-fusion variants with one- or combination of mutations were designed, which gained 5 to 1500 fold improved binding to FcRn at pH 6.0, and rates of transcytosis were shown to correlate with binding strength at both pH 6.0 and 7.4. Specifically, the mutant with most efficient apical to basolateral transcytosis, E505Q/T527M/K573P, showed 180 fold improved binding at pH 6.0 and only weak binding at pH 7.4. However, a mutant with 1,500 fold improved binding at pH 6.0 and strong binding at pH 7.4, E505Q/T527M/V547A/K573P, was transported poorly. Analysis of how selected mutants eluted from an human FcRn coupled column through a pH-gradient (pH 5.5-8.6), revealed that all mutants with improved binding to FcRn had distinct and longer retention time compared to WT in the following order; WT, K573P, V547A, V547A/K573P, E505Q/T527M/K573P and E505Q/T527M/V547A/K573P starting from pH 7.0 to 8.4. Interestingly, in the cellular transcytosis assay, transport was more efficient in the apical to the basolateral direction, and not affected by the presence of up to 120 fold excess IgG. This knowledge may pave the way for the development of albumin-fused drugs for efficient mucosal delivery.

General discussion and future perspectives

Albumin is the most abundant protein in serum, reaching 40 mg/ml in both human and mouse. In addition, albumin has a long serum half-life of three weeks, a feature that it shares with the unrelated protein, IgG. This common property is due to their interaction with the intracellular receptor, FcRn, which rescues both molecules from degradation via a cellular recycling pathway. Furthermore, albumin and IgG are present not only in serum, but also in various secretions at mucosal surfaces. While FcRn-mediated transcytosis into the mucus layer has been shown for IgG, this has not been demonstrated for albumin. The fact that FcRn can bind both albumin and IgG simultaneously in a non-cooperative manner *in vitro,* encouraged us to investigate the albumin-FcRn interaction as well as the importance of the interaction for cellular transcytosis. Furthermore, rodents are the first choice of animal models for pre-clinical screens, which emphasises the importance of investigating cross-species binding differences between human and murine molecules. We made recombinant proteins of albumin and FcRn, and tested their binding properties using SPR and ELISA. Different mutants of albumin and albumin fragments were designed; single- or combinations of domains and single- or combinations of amino acid substitutions in DI or DIII. Furthermore, selected DIII-mutants with altered binding at pH 6.0 and/or pH 7.4 were tested for their ability to be transcytosed across polarized human epithelial cells.

The albumin-FcRn interaction

Confirming previous reports, our binding studies show that recombinant albumin DIII binds more strongly to FcRn than the other two domains[92,95]. Moreover, during the work on this thesis, two co-crystal structures of the FcRn-albumin complex were reported[93,113]. One complex consist of human FcRn in complex with an engineered HSA variant with improved binding at both neutral and acidic pH, while the other contains WT HSA[93,113]. Furthermore, the complex containing WT HSA also contains an engineered human IgG1-Fc molecule with improved pH-dependent binding to FcRn, thus, demonstrating that the ternary complex of albumin-FcRn-IgG-Fc is formed *in* vitro[113]. Both reports confirmed that DIII gives the main contribution in the interaction[93,113].

A double mutant, K500A/H510Q, shows no measurable binding to FcRn. H510 and K500 are found in DIII of albumin and are situated in each end of a loop

connecting DIIIA and DIIIB (K500-H510). Inspection of the co-crystal structures of HSA bound to FcRn as well as HSA solved at pH 9.0 (pdb file 5IJF) suggests that FcRn W53 can only engage a hydrophobic pocket of DIIIB of albumin when this loop is stabilized by protonation of the conserved H535 and H510 at low pH[93,113]. In addition, H510 may also form an important stacking interaction to FcRn W176, and mutation to glutamine will likely disrupt this interaction. Furthermore, this double mutant will be important to include as a negative control in future studies. An illustration of the crystal structure of the IgG1-Fc-FcRn-HSAWT complex with close-ups of important areas and key residues involved are shown in figure 4.

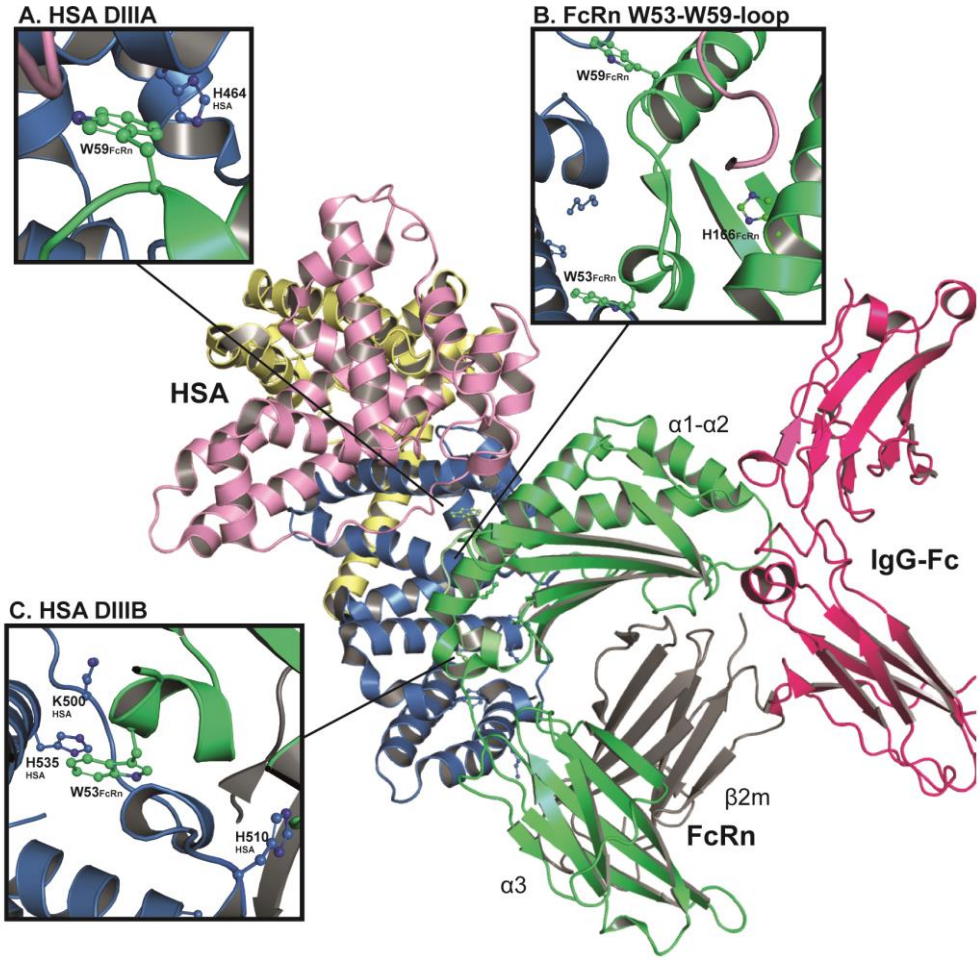

Figure 4. The co-crystal structure of the ternary complex of HSA, FcRn and IgG1-Fc-MST. The figure shows the solved structure of the ternary complex consisting of HSA, human FcRn and human IgG1-Fc-M252Y/S254T/T256E, MST. IgG1 Fc is shown in hot pink, while the HC (α1, α2 and α3) and β2m of FcRn are shown in green and grey, respectively. DI, DII and DIII of HSA are shown in pink, yellow and blue, respectively. **A-C.** Close-ups of the areas important for the FcRn-albumin-DIII interaction. **A.** FcRn W59 and HSA H464. **B.** The FcRn W53-W59-loop as well as H166. **C.** FcRn W53 and HSA H535, K500 and H510. Figures were made using the PyMol Software and the available crystal structure data[113].

It is now well accepted that DIII of albumin is the principle FcRn-binding domain, however, our studies have also revealed that two exposed loops in HSA DI contribute to optimal receptor binding. Specifically, SPR derived K_D values show that alanine substitution of N109, N111, L112 or P113 improve FcRn binding, while substitution of R81, E86, D89 and D108 decrease FcRn binding. These results support the hypothesis on the involvement of DI from the reported docking model, which is confirmed by the two solved co-crystal structures of HSA in complex with human FcRn[92,93,113]. However, the position where the amino acid substitution had the greatest negative influence on binding (D108A) does not form any critical intermolecular interactions, but instead D108 forms intramolecular hydrogen bonds with H105 of HSA DI and K466 of DIII, which then may stabilize albumin. In line with this, we show experimentally that a faster on- and off-rate for FcRn binding resulted when K466 was mutated to alanine, suggesting a role of this residue for optimal interaction with FcRn. Furthermore, the D108A mutation affected the α-helix content, as well as stability in serum, which hampered FcRn binding faster than other variants when incubated in serum. Based on these results, stability and correct positioning of the HSA DIII and DI relative to each other and FcRn, seems to be essential features of the DI-contribution.

Moreover, two of the over 60 reported naturally occurring albumin variants[114] were produced and tested for binding to FcRn, Vibo Valentia (E82K)[115] and Yanomama-2 (R114G)[116], and both showed 2 fold decreased binding to FcRn. The fact that these variants bind weaker will most likely result in shorter half-life in heterozygote individuals as they will be outcompeted by WT HSA for binding to FcRn.

The binding differences between human and mouse DI to FcRn has important implications for the choice of preclinical animal model used for testing of albumin variants and therapeutics. Investigation of the differences also gives clues as to how the interaction may be altered. Interestingly, our results show that MSA DIII binds strongly to human FcRn, which is not influenced by the presence of MSA DI. The presence of human DI on the other hand, increased MSA binding to both human and mouse FcRn, which is in line with earlier results showing improved binding to human FcRn compared to MSA when mouse DIII was swapped onto HSA[107]. Importantly, interdomain contacts may be affected when generating chimeric molecules. We demonstrate that the swapping of two residues in DI

between human and mouse that are involved in the DI-DII interface contacts affected binding to human FcRn, and surprisingly, improved binding was obtained in both cases. However, if these contacts are made in the MSA molecule remains to be addressed, as no solved crystal structure of MSA has been reported. Moreover, MSA DIII showed only 2 fold decreased binding to mouse FcRn, which is far from the more drastic reduction detected for HSA DIII towards human FcRn[92]. MSA DIII contains a proline at position 573, which contributes to strong binding[108]. Complete MSA thus acquires an optimal half-life. Circulating albumin is not only a carrier of nutrients, but also waste products like bilirubin and heme[117,118]. Hence, albumin half-life is functional optimized so as to maximize the distribution of nutrients without causing harm both in human and mouse.

We demonstrate that the K573P mutant can be combined with other mutations in DIII to create novel variants with considerably improved receptor binding. The effect of combination was surprisingly large, as combination with E505Q/T527M or V547A gave 180-200 fold improvement with retained pH-dependency, which is over 10 fold higher than ever reported for IgG engineering[73,97,102]. The K573P[108] and V547A[93] mutations may affect how the last C-terminal α-helix can engage binding to FcRn directly and indirectly, respectively, to facilitate strong interactions. Furthermore, inspections of the co-crystal structures show that both E505 and T527 in HSA are in close proximity to FcRn, and substitution of T527 to a more hydrophobic amino acid may facilitate stronger hydrophobic interactions with W53 of FcRn, while the removal of negative charges in HSA E505 may hinder repulsion from D231 of FcRn[93,113]. Moreover, combination of DIII and DI mutants may create novel binders with further improvement in pH-dependent binding kinetics. For instance, combining with a mutation of N111, which gave improved binding when substituting with alanine or serine could be of interest and such variants may result in improved half-life.

Albumin is a natural carrier of a plethora of insoluble and hydrophobic endogenous ligands, which binds to its hydrophobic pockets, its N-terminus or to the free cysteine[86,96,117–143]. Interestingly, its been shown that fatty acid binding induces conformational changes in albumin[86]. Furthermore, bound long-chain fatty acids negatively affect binding to FcRn[93]. How different ligands that bind to albumin affect FcRn binding and transport remains to be investigated.

Albumin biodistribution

From a therapeutic perspective, albumin is an ideal drug carrier due to its longevity, serum stability and body distribution. As such, strategies on utilizing albumin to improve pharmacokinetics of therapeutics are currently under development based on genetic fusion[107,108,144–166] chemical conjugation[167–178], association[144,179–197] or encapsulation into albumin-nanoparticles[198–209], as reviewed elsewhere[210,211]. Moreover, two albumin fused products were recently approved by FDA, both based on the Veltis® technology, which is the genetic fusion or chemical conjugation of drugs to recombinant albumin. Specifically, it is albumin N-terminally fused to either glucagon-like peptide-1 (Tanzeum)[212–214] or Factor IX (Idelvion)[215–218] for the use by patients with diabetes type 2 and haemophilia B, respectively. In both cases, a weekly injection is sufficient to sustain optimal levels in blood compared to multiple times a week when not fused to albumin. The diversity of molecules as well as strategies to generate albumin-based drugs encourages further development of even more optimal design. Basic research on how albumin is transported throughout the body may also open new possibilities for delivery.

We are the first to report that the monovalent FcRn ligand, albumin, can be transcytosed across polarized human intestinal epithelial cells. Efficient transport depends on endosomal acidification and binding to FcRn, as disruption of endosomal acidification, blocking of the albumin-binding site on FcRn or usage of the FcRn-disabled binder (K500A/H510Q) hampered transport. This opposes a previous report using rat FcRn transfected MDCK cells where FcRn-dependent transport of rat albumin was not detected[78]. Furthermore, if these different outcomes are due to the use of different cell lines or cross-species differences remain to be investigated. On the other hand, the same report showed that bivalent binding of IgG-Fc to FcRn was not required for transport[78], which is supported by our findings. Furthermore, high amounts of either FcRn-ligand do not affect transcytosis of the other. However, whether the receptor is saturated or not in this assay is not known. Thus, proof of the formation of ternary complexes in a cellular setting is yet to be shown. All in all, the findings encourage further investigation of albumin transcytosis at mucosal surfaces *in vivo* and the importance of FcRn. How albumin are distributed at mucosal surfaces may lead to the development of next-generation albumin-based drugs for mucosal delivery.

That transport of albumin is more efficient in the apical to basolateral direction is an interesting finding, as the opposite has been shown for human IgG using the same cell type[30,63]. Biologically, the basolateral to apical transport of IgG has been shown to be important in immune surveillance and protection at mucosal surfaces by the sampling of luminal antigens, followed by transcytosis of the formed IgG-ICs back to the lamina propria in mice, which in all cases are dependent on FcRn[31,67,219,220]. Furthermore, the cellular distribution of the receptor as well as the directional transport of IgG have been shown to differ between human and rodent cell lines, as the rat version is localized to the apical side and transport to the basolateral side, which is the opposite found for the human receptor[60,62,63,74,75]. Differences in *N*-glycosylation of FcRn HC between human and rat may explain these observations[10,221]. However, the human throphoblast BeWo cell line has been shown to have a more efficient apical to basolateral direction of IgG transcytosis[65,222], which suggests not only a species dependent, but also cell type dependent directional transport, which likely reflects the function of the receptor in different tissues and organs. The directional transport of albumin may be an ideal gateway for efficient non-invasive delivery of albumin-based drugs.

The FcRn coupled column has recently been used to investigate FcRn binding properties of IgG variants as a function of pH (pH 5.5-8.6), and a linear correlation between column retention time and half-life was observed for the variants tested[223,224]. We successfully adopted this method for the separation of albumin variants based on their pH-dependent binding properties. The long retention time (pH 8.4) of E505Q/T527M/V547A/K573P may explain its low transport efficiency as its probably restrained inside cells, and the elution pH of E505Q/T527M/K573P (pH 8.0) may represent a threshold in regard to retention time without affecting transcytosis. However, if this translates to an *in vivo* setting is important to investigate. For IgG engineering, the threshold K_D value for binding to FcRn at neutral pH without affecting recycling has been investigated[225]. For the mutants tested, 1.1 μM was the lowest K_D value before decreased half-life was seen[225]. However, if this threshold value also applies for albumin and/or in general for all engineered IgGs remains to be investigated. In addition, how binding strength at pH 7.4 for both ligands affects recycling and transcytosis remains to be determined. Engineered albumin variants with enhanced rate of transcytosis may

be more efficiently transported than WT albumin *in vivo*, and thus, may be ideal carriers of drugs for delivery across mucosal barriers.

Examination of how different albumin fusions bind FcRn is important to consider as alterations may affect the pharmacokinetics *in vivo*. Interestingly, a shift in the elution profile from pH 6.5 to 7.0 on the FcRn coupled column was observed when albumin was fused to glutathione-s-transferase (GST), which indicates that the fusion partner can affect FcRn binding through a pH gradient. In line with this, reports on albumin (WT and K573P) fused to scFv showed that fusion to either the N- or C-terminal end did not greatly impact binding to FcRn, although a minor reduction in binding was seen for the C-terminal fusion[107,108]. Moreover, it is an interesting finding that unfused HSA elutes from the FcRn coupled column at pH 6.5, which is more acidic than that reported for IgG (pH> 7.3)[224]. What this means biologically remains to be investigated. A higher dissociation pH may potentially result in more efficient uptake by binding to FcRn transiently exposed on cell surfaces.

Furthermore, mucosally delivered Fc-fused drugs and vaccines have been shown to be successfully taken up in an FcRn-dependent manner by transcytosis of the intact fusion or Fc-conjugated nanoparticles across epithelial cells *in vitro* and *in vivo*[27,31,63,68–71,226–229]. For instance, the fusion of erythropoietin (EPO) or follicular stimulating hormone (FSH) to Fc was taken up in a FcRn-dependent manner after pulmonary delivery in humans and/or cynomolgus monkeys, respectively[68,69,226,230]. Notably, comparison of different fusion formats of EPO-Fc and FSH-Fc was shown to affect uptake and activity, respectively[69,226], which emphasize the importance of testing FcRn binding and transport properties for each unique fusion[63,231]. Albumin variants with improved FcRn binding and transport properties may be ideal fusion partners as the engineered variants may compensate for negative influence upon fusion or conjugation and thus compete more favourably with endogenous albumin.

Targeting FcRn for the mucosal delivery of albumin fusions

We demonstrate that albumin fusions can indeed be transcytosed across polarized human epithelial cells *in vitro*. Previously, promising results have been obtained for delivery of IgG Fc-fused drugs and vaccines across mucosal barriers[27,31,63,68–71,226], and albumin-fusions may be delivered in a similar manner. Interestingly, a recent study used albumin as a target for a subunit vaccine, where

adjuvant or antigen was coupled to lipophilic tails that naturally bind hydrophobic pockets of endogenous albumin[232]. These vaccine constructs were injected subcutaneously in mice where the vaccine constructs were shown to accumulate in lymph nodes, and as such generate robust immune responses[232].

Furthermore, it is well characterized how IgG-ICs are handled by monocyte derived dendritic cells, and that the cross binding of classical Fcγ-receptors (FcγRs) followed by binding to FcRn are crucial for efficient antigen presentation by MHC class I or II, which subsequently induce T cell responses[56,233]. This FcRn-dependent enhancement in degradation and subsequent antigen presentation may also occur for Fc-fused vaccines upon binding to antibodies raised against the fused antigen, although not shown experimentally[70,71]. Importantly, the size of IgG-ICs are suggested to be important for the trafficking to the degrading pathway in both monocyte derived dendritic cells and endothelial cells, which in the latter case is shown to be dependent on cross binding of FcRn molecules and not its cytoplasmic tail[56,233,234]. By a similar mechanism, trafficking of the transferrin receptor is modulated by ligand valence[235]. However, the size limit of the IgG-IC for trafficking to the degrading pathway in different cell types, and if this also applies for Fc-fusions is not yet known.

This encourages further studies on how mucosal delivered albumin-based vaccines are handled by lamina propria resident dendritic cells, as well if such constructs can home to lymph nodes for induction of efficient immune responses. We do have preliminary data of nasal delivered MSA (WT and K500A/H510Q) fused to hemagglutinin from Influenza A together with CpG as adjuvant to conventional mice, and robust antigen-specific antibody responses were only induced for the WT construct, which also gave 100% protection after administrating a lethal dose of the corresponding virus (*Bern et al, manuscript in preparation*). This suggests that albumin-antigen fusions are (1) transcytosed across the nasal epithelia, and (2) taken up and processed by immune cells for the generation of immune responses in mice, and that efficient induction of antigen-specific antibodies depends on FcRn binding.

Our *in vitro* studies and preliminary *in vivo* data motivate us to explore the use of albumin as a carrier of drugs and vaccines. Such proof of concept studies should be performed by comparing the albumin strategy with the Fc technology as to compare their ability to induce robust immune responses. Interestingly, it has

been shown that prolonged and increased exposure to the antigen enhances antibody responses[236]. Using albumin as a fusion partner, the antigen may sustain longer in the body than conventional subunit vaccines, and thus enhance protective immunity. Interestingly, engineered variants with improved half-life or properties that enable faster trafficking to the processing pathway may be able to enhance immune responses. Furthermore, conjugating an adjuvant to for instance C34 of albumin could also ensure co-delivery of both adjuvant and antigen to the same cell. Another possibility could be to deliver albumin fusions in the absence of adjuvant for induction of T-regulatory cells, intended as a tolerating or desensitization vaccine. If such a strategy works, the concept may be tested for a wide range of allergens and drugs, which induce allergic reactions and anti-drug antibodies, respectively. An illustration on the mucosal delivery approach for albumin-fused vaccines is shown in figure 5.

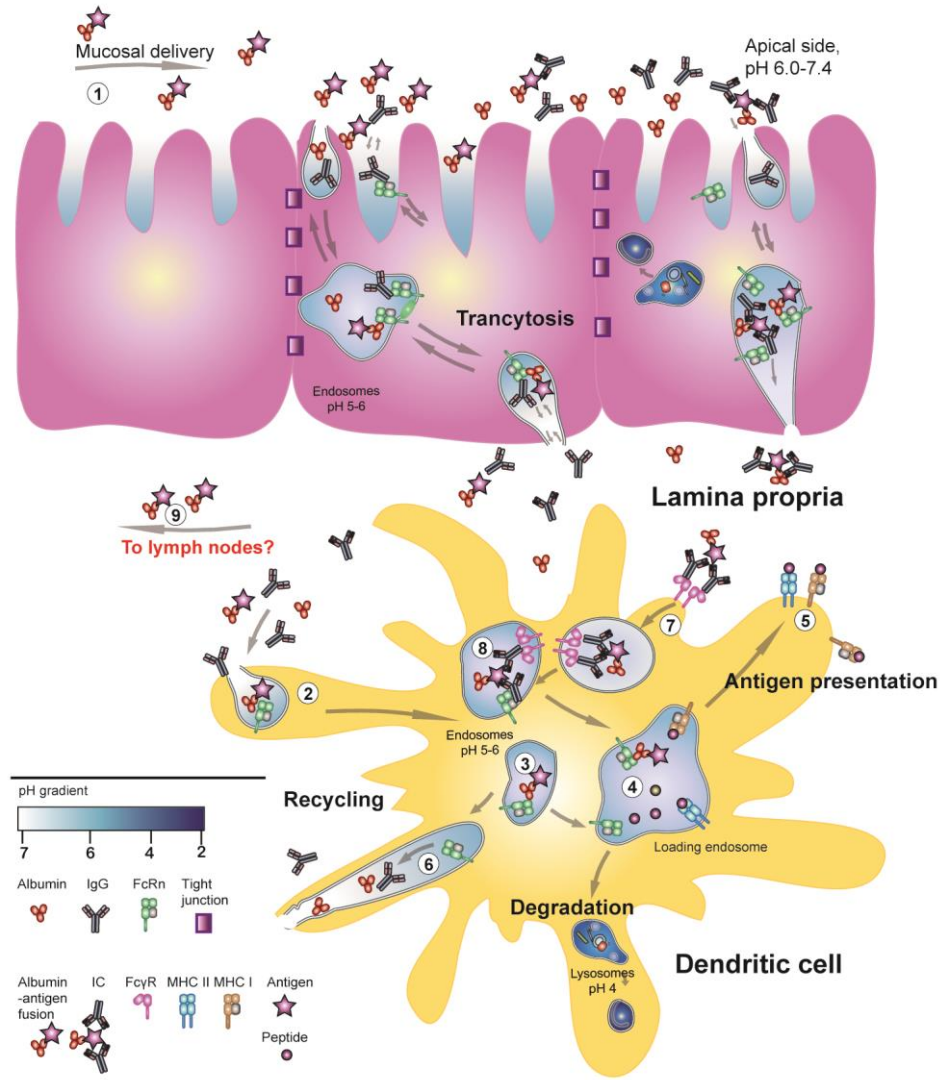

Figure 5. Delivery of albumin-antigen fusions across mucosal barriers (1) Albumin-antigen fusions are administered to a mucosal site together with adjuvant (not shown), and polarized epithelial cells lining the lumen take up the fusions followed by binding to FcRn inside acidified endosomes, which can transcytose the fusions across the cellular layer for release into the lamina propria in a pH-dependent manner. It may also be possible that binding to FcRn causes recycling back to the luminal side. Mucosal resident IgG as well as albumin will be present at the luminal side and may compete for binding to FcRn inside endosomes. Transcytosed albumin fusions may then be taken up by immune cells such as macrophages or dendritic cells (2), followed by trafficking to sorting endosomes where it can encounter FcRn (3) for subsequent processing for antigen loading on MHC molecules (4) and presentation to T cells (5). Dendritic cells are also capable of recycling monomeric IgGs and albumin to the plasma membrane for their exocytosis (6). The resulting antibodies raised against the albumin-antigen fusions will bind persistent fusions and form multivalent ICs that can cross-bind FcγRs on dendritic cells (7) for the sequential hand-off to FcRn (8). FcRn can traffic the ICs to the loading compartment where antigen-derived peptides can be generated followed by binding to the grooves of MHC class II or I (cross-presentation) (4). Peptide-loaded MHC molecules can then traffic to the cell surface for the presentation to T cells (5). Alternatively, albumin-antigen fusions are directly transported to the lymph nodes by the circulation (9).

In line with previous reports[29,31,237,238], we demonstrate expression of FcRn in tissues derived from the human intestine, rectum and vaginal tract, and such sites may be a gateway for oral, rectal or vaginal non-invasive delivery of albumin-based drugs. However, a major challenge for such delivery is to maintain resistance to the proteolytic and acidic environment, especially in the gut. *In vivo* studies in cynomolgus monkeys have shown that orally delivered IgG obtained only low systemic levels[239]. However, IgG-Fc-coated nanoparticles have been shown to be more effective upon gavage delivery in mice[228,240]. This encourages further investigation of albumin-based nanoparticles for oral delivery, as albumin is highly stable, do not bind FcγRs, and as drugs can be coupled to C34 or associated by its hydrophobic pockets. Alternatively, nanoparticles coupled to albumin C34 may retain its FcRn binding properties via DIII.

Next-generation mouse models for the evaluation of novel human albumin variants and albumin-based drugs are required for the efficient screening and identification of candidates with superior pharmacokinetics before testing in larger animals. The optimal rodent model available for the investigation of human albumin-based drugs without competition and under the influence of FcRn are mice expressing the human FcRn HC under control of the human promoter and that in addition are knock out for MSA (Tg32-albumin-/- mouse FcRn-/- human FcRn +/+)[241]. The half-life of intravenously injected HSA was shown to be 24 days in these mice, and pre-loading of HSA can also be done to address the effect of competition[241]. Another novel strain that has been developed, is knock out for both mouse FcRn and albumin, and knock in for the human counterparts under control of the endogenous mouse promoters (human FcRn+/+, human albumin +/+)[242]. Different tissue distribution of the receptor compared to human and the competition effect from high levels of circulating HSA makes this a very different model, and the half-life of HSA in this model was found to be 50 hours after intravenous administration[242]. However, both mouse models will have low levels of endogenous IgG as human FcRn binds only weakly to mouse IgG subclasses[109,110]. These new mouse models should be used for evaluation of HSA-based drugs and vaccines. Alternatively, an additional knock in for human IgG1 Fc could be attractive, as human IgG1 has been shown to bind all mouse FcγRs, and was found to be the most potent human IgG isotope in mouse tumor xenograft models[243]. Furthermore, engineered human albumin variants with improved

binding to FcRn that are able to compete effectively with endogenous MSA could be used in WT mice but also in the new strains as to enhance transcellular transport and half-life.

Moreover, investigation on how other albumin receptors such as cubilin and megalin are affecting albumin biodistribution is highly needed. Megalin and cubilin have been found to be co-expressed on apical surfaces of epithelial cells from several tissues and species including mouse, rabbit, rat, canine, dog and human[47,48,50,244–258], and are involved in endocytosis of several ligands, including albumin. Both are shown to be important in albumin re-absorption in the kidneys[45–50], where also FcRn has been shown to be involved[43,44]. This emphasizes the importance of investigating how engineered albumin variants with altered FcRn binding affect the handling by cubilin and megalin. Currently there are no reports on which domain(s) of albumin that are involved in interactions to cubilin and megalin, as well as if binding to either interfere with FcRn binding and functions.

Methodological considerations

Production of recombinant proteins

In this study, recombinant FcRn and albumin have been produced. In all cases, cloned constructs have been sequenced before production, and the integrity of the proteins analysed on sodium dodecyl sulfate gels before they were used in further experiments. In Paper I, GST-tagged human FcRn was produced transiently in adherent Human Embryonic Kindey (HEK) cells, as previously described[259]. HEK cells are able to attach *N*-glycans on the glycosylation site(s) of FcRn, and the proteins derived from this system have been used successfully in other published studies[40,92,94,109,194]. In addition, mouse and human FcRn with a C-terminal his-tag was used in Paper I-III, which was produced in insect cells following a previously described procedure[12,260,261]. Size-exclusion chromatography to eliminate aggregations or impurities was critical to maintain the receptors as monomeric fractions. Unfused albumin was used in Paper I-II, which was produced in yeast or HEK cells. However, as albumin is not *N*-glycosylated, no major functional differences are expected between the preparations. For Paper I-III, most engineered albumin variants were produced with a C-terminal GST-tag, and no major differences were seen between the variants in regard to the levels secreted from the cells and yield after purification. In addition, the GST-tagged albumin variants were analysed using circular dichroism spectroscopy to address their secondary structural elements and stability at different temperatures or at 25 degrees only. The GST-tagged proteins were constructed to have a glycine-serine-linker between albumin and GST to allow flexibility and minimize the possibilities for that the GST-tag could affect binding to FcRn.

ELISA

ELISA is a rapid and sensitive method to measure protein interactions. Prior to all experiments, albumin variants to be compared were normalized using ELISA and anti-albumin and/or anti-GST antibodies. Titrated amounts were added in duplicates and concentrations were adjusted until similar absorbance values were obtained for all variants. Normalization was then confirmed by SDS-PAGE analysis. In Paper I-III, to investigate binding of different albumin variants to FcRn, an engineered human IgG1 variant (MST-HN) that bind strongly to both human and mouse FcRn at both pH 6.0 and 7.4 was coated in wells, followed by adding

his-tagged FcRn, GST-tagged albumin and horseradish peroxidase-conjugated anti-GST from goat. This set up was chosen as we know that IgG does not interfere with albumin binding to FcRn *in vitro*[40,41], and anti-his antibodies would not work at pH 6.0 due to protonation of histidines below pH 7.0. Most importantly, this set-up gave a highly sensitive read out compared to other anti-FcRn antibodies or nanobodies that have been tested and works well for screening of albumin variants to both the human and mouse forms of FcRn. In contrast to human, the mouse receptor binds strongly to most species of IgG[110]. However, the bound mouse receptors will have the IgG-Fc binding sites occupied by binding strongly to the MST-HN variant and will not be able to bind the detection antibody. ELISA cannot measure kinetic values describing on- and off-rates, and SPR was done to obtain a better understanding about the binding kinetics.

SPR

SPR was used to determine the binding kinetics between albumin and FcRn. GST-tagged FcRn and albumin was randomly immobilized on a CM5-chip by amine coupling, followed by injection of unfused albumin and his-tagged FcRn, respectively. GST-tagged proteins may show a small degree of aggregation that is due to the presence of free cysteines[262,263], and injection of GST-fusions may cause re-binding effects that can affect the kinetic calculations. However, coupling of GST-fusions is achievable. Low levels of protein were coupled to avoid mass transfer effects or steric hindrance. The random coupling procedure used may interfere with functionality, but in our case, the proteins remained functional after immobilization, and a similar distribution of proteins with available binding sites on all chips should allow comparison of engineered variants. The binding constants achieved may differ between assay set ups, but the hierarchy and the fold differences between variants should be reproducible. As such, the comparison of K_D values should only be done within the same set up.

Importantly, monomeric molecules of the injected fractions are essential to calculate accurate binding constants. Monomeric fractions of unfused albumin, his-tagged mouse and human forms of FcRn were gel filtrated right before SPR analyses, as to avoid artefacts due to aggregations. Notably, minor aggregates of the receptor fractions have been observed after 3 weeks storage at 4 degrees after one round of gel filtration. As the stoichiometry of the albumin-FcRn interaction is 1:1, the binding kinetics were estimated using the Langmuir 1:1 ligand

binding model provided by the BIAevaluation 4.1 software. Importantly, non-specific binding and bulk buffer effects were corrected by subtracting the obtained binding responses from the control CM5 flow cells and blank injections.

FcRn affinity chromatography

In Paper III, a human FcRn coupled column was used to investigate the binding properties of albumin fusion variants as a function of pH (pH 5.5-8.6). The assay has previously been established to study the binding properties of IgGs, and we here adopted the assay to study albumin variants. The column has been made by biotinylation of recombinant FcRn HC on its C-terminal AviTag followed by mixing it with a solution of streptavidin coupled sepharose[223,224]. Injection of ligands over the column through a pH gradient mimics the gradual change in pH during exocytosis. However, due to the high density of coupled FcRn compared with what is found within endosomal compartments, re-binding may occur and result in a retention time longer than what expected in a cellular setting. However, this is a highly sensitive method that can be used to distinguish between engineered variants and reveal minor but important differences in FcRn binding properties through a pH gradient.

The application of pH gradient that reach a slightly basic value manages the generation of distinct peaks for all variants tested. It could be possible to terminate the increase at pH 7.4 and let the proteins detach over time. However, this would make the dissociation times longer for the variants with affinity at pH 7.4, which most certainly will generate broad detectable peaks. Control experiments were done with an albumin mutant (K55A/H510Q) with no measurable binding to FcRn, and this mutant went straight through the column, which demonstrates that albumin does not bind the sepharose material.

Transwell

In Paper III, a transcytosis assay using the T84 cell line was adopted[63] to investigate albumin transcytosis. This cell line is from cancerous colon tissue and expresses endogenous FcRn, where the level of expression was shown to be similar to that found in normal human colon tissue, as revealed by confocal microscopy using a monoclonal anti-human FcRn antibody. However, whether the cancerous derived cell line has any gene alterations that may affect transcytosis are not known.

This is a model assay to predict albumin or IgG transcytosis, but the set up on filters does not directly reflect the milieu that are present on the mucosal surfaces *in vivo*. Specifically, mucus lining mucosal surfaces may affect the uptake of proteins *in vivo* by retain proteins longer at the apical side by making a thicker barrier to pass through. Routinely, we bought fresh cells from the providing company and tested them for mycoplasma. Furthermore, the different morphology and cell division speed between this cell line and HEK cells make the chances for cell contamination unlikely. Low passage numbers were always used and cells were carefully seeded as single cells onto filters and polarization was monitored by resistance measurements. Moreover, media used during all experiments were always warmed to 37 degrees, as low temperature has been shown to hamper transcytosis[30]. As the growth medium contains bovine serum albumin and IgG, the cells were washed and starved for 1 hour using Hanks Balanced Salt Solution to eliminate possible interference of the bovine-derived proteins. This salt solution does not allow the cell layer to retain its integrity for more than 5-6 hours due to the lack of nutrients, and as such we chose 4 hours incubation time and checked that the resistance in each well remained the same after each experiment. Different incubation periods and medium compositions may be adjusted in the future to obtain longer incubation times. As such, media composition but also the pH may affect the cells and should be monitored carefully.

Monoclonal antibodies directed against human IgG and albumin were used to establish sensitive ELISAs to determine the amounts of proteins transported across the cell layers. Importantly, the anti-albumin antibodies used were not affected by DIII engineering of albumin. Titrated amounts of the proteins used in the cellular assays were included in the ELISAs as standards. The sensitive ELISAs established exclude the need for labelling of the proteins added, which may interfere with transport and FcRn binding. The assay was used to address how engineered albumin variants with different FcRn binding properties were transported across the cells, which revealed distinct capacities to be shuttled from the apical to the basolateral side. As such, the assay is an attractive tool for screening of albumin variants and fusions prior to *in vivo* evaluation.

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